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Identification of carbohydrates on the surface membrane of pathogenic and nonpathogenic piscine haemoflagellates, *Cryptobia salmositica*, *C. bullocki* and *C. catostomi* (Kinetoplastida)

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ABSTRACT: Carbohydrates and protein glycoconjugates on the cell membranes of *Cryptobia salmositica*, *C. bullocki* and *C. catostomi* were analyzed using 13 highly purified lectins (unlabelled or digoxigenin/biotin labelled). No agglutinations were observed with *C. salmositica*, *C. bullocki* and *C. catostomi* using lectin TPA (*Tetragonolobus purpureas* agglutinin, for α-L-fucose). *C. salmositica* was agglutinated by 3 of 12 lectins [Con A, for α-man and α-D-glc; PSA, for α-man; PWM, for (glcNAc)3], while *C. bullocki* was agglutinated by 8 lectins and *C. catostomi* was agglutinated by 10 lectins. Glycoconjugate analysis with digoxigenin or biotin labelled lectins showed a species-specific staining pattern in pathogenic and nonpathogenic *Cryptobia* spp. The nonpathogenic *C. catostomi* had the strongest reaction. These results indicate that the surface carbohydrate residues and glycoconjugate compositions on *Cryptobia* spp. are different between species; they may be related to the virulence of the parasite.

KEY WORDS: *Cryptobia salmositica* · *C. bullocki* · *C. catostomi* · Surface carbohydrates · Glycoconjugates · Fish · Haemoflagellates

INTRODUCTION

The plasma membrane of parasitic protozoa is heavily glycosylated (Dawidowicz et al. 1975, Dwyer 1977, Schottelius & Muller 1984, Wilson & Pearson 1984, Nagakura et al. 1986, Ghosh et al. 1988). These glycoconjugates, which are involved in parasite survival or infectivity, are potential targets for the development of chemotherapeutic agents. Since different carbohydrate residues appear to be involved in the glycoconjugated function, the identification of these residues may yield new insights into membrane architecture. Lectins, a class of carbohydrate-binding proteins or glycoproteins, are useful for elucidating similarities and differences in the surface architecture of cells. Lectins have been used to distinguish infective and noninfective amastigotes and promastigotes of *Leishmania major*, *L. mexicana amazonensis*, *L. braziliensis* and *L. enriettii* (see Dawidowicz et al. 1975, Sacks et al. 1985, Saraiva & Andrade 1986, Schottelius 1987), and to identify the membrane glycoconjugates in *Leishmania* species and strains of African trypanosomes (Mutharia & Pearson 1987, Rossell et al. 1990, Jaffe & McMahon-Pratt 1988). Lectins have also been used to reveal differences in freshwater and marine fish trypanosomes (Zajicek & Peckova 1990, Zajicek & Lukes 1992).

Cryptobia salmositica causes salmonoid cryptobiosis and it has been found in all species of Pacific salmon on the west coast of North America (Woo 1987, 1994). The clinical signs include exophthalmia, general oedema, splenomegaly, abdominal distention with ascites, and anorexia (Woo 1979, Li & Woo 1991). *C. salmositica* causes mortality in experimentally and naturally infected salmonids (Woo 1987) and it has also been identified as a lethal pathogen in semi-natural and intensive salmon culture facilities on the Pacific coast of

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North America (Bower & Thompson 1987). *C. bullocki* is a pathogenic haemoflagellate in summer flounders *Paralichthys dentatus* on the Atlantic coast of North America and the Gulf of Mexico (Burreson 1982). Clinical signs include anaemia, general oedema and splenomegaly. *C. catostomi* is a nonpathogenic haemoflagellate in white suckers *Catostomus commersoni* in southern Ontario, Canada (Bower & Woo 1977a, b). Characterization of cell surface carbohydrates may allow a better understanding of differences between the pathogenic and nonpathogenic species of *Cryptobia*.

The main objective of the present study was to identify cell surface carbohydrates of *Cryptobia salmositica*, *C. bullocki* and *C. catostomi* using 13 highly purified lectins.

MATERIALS AND METHODS

Parasites. All parasites, *Cryptobia salmositica*, *C. bullocki*, *C. catostomi*, were cultured as described earlier (Feng & Woo 1996). They were harvested by washing 3 times at 4°C (centrifugation at 10 000 × *g* for 15 min each time) in cold-blooded vertebrate Ringer's solution (CBVR) and resuspended in cold CBVR. The number of parasites was determined using a haemocytometer (Archer 1965).

The pathogenic *Cryptobia salmositica* was isolated from the blood of an infected rainbow trout *Oncorhynchus mykiss* and cultured in a modified minimum essential medium at 12°C for no more than 2 mo as previous studies have shown that the parasite retained its pathogenicity in the short-term *in vitro* culture (Woo & Thomas 1991).

Surface membrane extraction. The surface membrane of the parasites was prepared as previously described (Feng & Woo 1996). Briefly, after the final wash, parasites were lysed in Ringer's solution containing 0.5% Triton X-100, 2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM phenylmethylsulphonyl fluoride (PMSF) and 100 µg ml^{-1} leupeptin, vortexed for 10 s, then centrifuged at 7000 × *g* for 10 min at 4°C after the last washing. The supernatant containing dissolved protein was saved as surface membrane protein. Total protein concentration was determined according to Bradford (1976) and the sample was stored at –100°C.

Enzyme treatment (Pereira et al. 1980). Washed intact live parasites (1×10^8 ml^{-1}) were mixed with an equal volume of trypsin in 50 mM Tris-HCl, pH 7.5 to give final concentration of 1 mg ml^{-1}, and incubated in an incubator for 60 min at 10°C.

For the neuraminidase treatment the parasites were resuspended in phosphate-buffered saline (PBS), pH 6.0 containing 0.1 to 0.2 U of *Vibrio cholerae* neuraminidase (Boehringer Mannheim Canada, 201 Boulevard Armand Frappier, Laval, Quebec H7V 4A2, Canada) and incubated at 10°C for 60 min.

After treatment, the cells were washed once with 15% trichloroacetic acid (TCA) to inactivate the enzyme, washed 3 times with chilled PBS, and resuspended at a suitable concentration for agglutination. The negative controls (for enzyme digestion) received the same amount of buffer in place of enzymes. All treatments were in triplicate.

Lectin-mediated agglutination. Lectins (Table 1) were obtained from Sigma (Sigma-Aldrich Canada Ltd, 2149 Winston Park Drive, Oakville, Ontario L6H 6J8, Canada). Parasites were washed 3 times in PBS, pH 7.4. Agglutination assays were performed in 96-well, flat bottomed microtiter plates. Lectins were adjusted to final concentrations in PBS and studied alone or with 10 mM inhibitory sugar. Parasites were added to produce a final concentration 5×10^7 ml^{-1} in a 200 µl volume and the mixture of parasites and lectin was left at 10°C for 1 h. Agglutination was determined under inverted microscope and in each experiment a blank (without lectin) was used as control. In the tables (–) indicates no agglutination, (+) indicates agglutination with a few clumps, (2+) indicates one-third of the parasites were agglutinated, (3+) indicates two-thirds of the parasites were agglutinated, (4+) indicates all parasites were agglutinated.

SDS-PAGE and lectin blot. The terminal sugars of glycoproteins on the surface membrane were determined using digoxigenin labelled lectins and Biotin labelled lectins. Surface membrane protein from cryptobiids was separated using the discontinuous gel

Table 1. Lectins used in this study

Lectin	Source	Binding specificity	Labelling
Con A	*Canavalia ensiformis*	α-man, α-glc	Biotin
LCA	*Lens culiaris*	α-man	Biotin
PSA	*Pisum sativum*	α-man	Biotin
PCA	*Phaseolus coccineus*	α-D-man	Biotin
GNA	*Galanthus nivalis*	non-reduc.α-(1-3), α-(1-6),α-(1-2)man	Digoxigenin
HPA	*Helis pomatia*	galNAc	Biotin
WGA	*Triticum vularis*	(glcNAc)2,neuNAc	Biotin
PWM	*Phytolacca americana*	(glcNAc)3	Biotin
TPA	*Tetragonolobus purpureas*	α-L-fucose	Biotin
ECA	*Erythrina cristagalli*	β-gal(1-4)glcNAc	Biotin
PNA	*Arachis hypogaea*	β-gal(1-3)galNAc	Digoxigenin
SNA	*Sambucus nigra*	α-NeuNAc(2-6)gal/galNAc	Digoxigenin
MAA	*Maackia amurensis*	sialic acid α-(2-3)gal	Digoxigenin

system of Laemmli (1970) and transferred to the nitrocellulose membrane (Towbin et al. 1979). Strips with protein were blocked with a blocking reagent for 1 h, washed with Tris-buffered saline (TBS), incubated subsequently with digoxigenin-labelled or biotin-labelled lectins, and then with anti-digoxigenin or streptavidin, both of them conjugated with alkaline phosphatase for 1 h. Finally, the strips were incubated with substrate to develop a grey to almost black colour to visualize glycoproteins with terminal sugar.

Deglycosylation (glycosidases digestion). Deglycosylation was performed using western blot. The protein transferred onto the nitrocellulose was digested with N-glycosidase F (dissolved in a buffer containing 0.1 M sodium phosphate, 10 mM EDTA, 0.5% Triton X-100, 0.05% SDS and 1% 2-mercaptoethanol), endoglycosidase H or endoglycosidase F for 60 min at 37°C. Blotted protein was also treated with neuraminidase (from *Arthrobacter ureafaciens* in 0.1 M sodium acetate buffer, pH 5.0), galactose oxidase (from *Dactylium dendroides* in 100 mM potassium phosphate buffer at pH 6.0) and α-mannosidase (from *Canavalia ensiformis* in ammonium sulphate solution, pH 6.0) sequentially to determine the type of N-glycan. The enzymes were inactivated with 15% of TCA for 30 min at room temperature. The strips of nitrocellulose then were subsequently blocked with 0.5% gelatin, incubated with lectin from *Galanthus nivalis* (GNA), then anti-digoxigenin enzyme conjugate and substrate. Negative control strips for enzyme digestion received the same amount of buffer for incubation in place of glycosidase. All treatments were in triplicate.

RESULTS

Differences between pathogenic and non-pathogenic *Cryptobia* spp. in lectin-mediated agglutination

Comparison of the lectin-mediated agglutination of *Cryptobia salmositica*, *C. bullocki* and *C. catostomi* showed there were 2 lectins (Con A and PSA) that agglutinated the 3 species. However, different concentrations of PSA were required: 8 μg ml^{-1} for *C. salmositica*, 64 μg ml^{-1} for *C. bullocki* and 32 μg ml^{-1} for *C. catostomi*. *C. salmositica* was agglutinated by only 3 lectins (Con A, PWM and PSA). *C. bullocki* was agglutinated by 8 lectins (Con A, ECA, HPA, LCA, PCA, SNA, WGA and PSA) and *C. catostomi* was agglutinated by 10 lectins (Con A, ECA, HPA, LCA, PCA, PWM, SNA, MAA, PNA and PSA). Seven lectins (Con A, ECA, HPA, LCA, PCA, SNA and PSA) agglutinated both *C. bullocki* and *C. catostomi* and a lower concentration of all 7 lectins was required by *C. catostomi* than by *C. bullocki* for agglutination. MAA and PNA only agglutinated *C. catostomi*, but WGA only clumped *C. bullocki*. TPA did not agglutinate any of the *Cryptobia* spp. (Table 2).

The agglutination of pathogenic *Cryptobia salmositica* with some lectins was enhanced after enzyme treatment (Tables 3 & 4). ECA, HPA, LCA, SNA, WGA and PSA agglutination were enhanced and PWM agglutination decreased after neuraminidase treatment. ECA, PWM-mediated agglutination was removed and Con A, PSA-mediated agglutination was decreased after trypsin treatment. However, LCA-mediated agglutination of *C. salmositica* was enhanced after trypsin treatment (Table 4).

Table 2. *Cryptobia salmositica* (Cs), *C. bullocki* (Cb), and *C. catostomi* (Cc). Agglutination by lectins. (–) no agglutination; (+) agglutination with a few clumps; (2+) one-third of parasites agglutinated; (3+) two-thirds of parasites agglutinated; (4+) all parasites agglutinated

Lectin	Parasite species	Lectin concentration (μg ml^{-1}) 0.0	1.0	2.0	4.0	8.0	16	32	64	128	256
Con A	Cs	–	+	+	+	2+	2+	3+	3+	4+	4+
	Cb	–	+	+	+	2+	3+	4+	4+	4+	4+
	Cc	–	+	+	+	2+	3+	3+	4+	4+	4+
ECA	Cs	–	–	–	–	–	–	–	–	–	–
	Cb	–	–	–	–	–	–	–	+	+	+
	Cc	–	+	+	+	+	+	+	+	+	+
HPA	Cs	–	–	–	–	–	–	–	–	–	–
	Cb	–	–	–	–	–	–	–	+	+	+
	Cc	–	–	+	+	+	2+	2+	3+	3+	3+
LCA	Cs	–	–	–	–	–	–	–	–	–	–
	Cb	–	–	–	–	–	–	–	+	+	2+
	Cc	–	–	+	+	+	+	2+	2+	2+	2+
PCA	Cs	–	–	–	–	–	–	–	–	–	–
	Cb	–	–	–	–	–	–	–	–	–	+
	Cc	–	–	–	–	–	+	+	+	+	+
PWW	Cs	–	–	–	–	–	–	–	–	–	+
	Cb	–	–	–	–	–	–	–	–	–	–
	Cc	–	–	–	+	+	+	+	2+	2+	3+
SNA	Cs	–	–	–	–	–	–	–	–	–	–
	Cb	–	–	–	–	–	–	–	–	–	+
	Cc	–	–	–	+	+	+	+	+	2+	2+
TPA	Cs	–	–	–	–	–	–	–	–	–	–
	Cb	–	–	–	–	–	–	–	–	–	–
	Cc	–	–	–	–	–	–	–	–	–	–
MAA	Cs	–	–	–	–	–	–	–	–	–	–
	Cb	–	–	–	–	–	–	–	–	–	–
	Cc	–	–	–	–	–	+	+	+	+	2+
WGA	Cs	–	–	–	–	–	–	–	–	–	–
	Cb	–	–	–	–	–	–	–	–	+	+
	Cc	–	–	–	–	–	–	–	–	–	–
PNA	Cs	–	–	–	–	–	–	–	–	–	–
	Cb	–	–	–	–	–	–	–	–	–	–
	Cc	–	–	–	–	–	–	–	–	+	+
PSA	Cs	–	–	–	–	+	+	+	2+	2+	3+
	Cb	–	–	–	–	–	–	–	+	+	+
	Cc	–	–	–	–	–	–	+	+	2+	3+

Table 3. *Cryptobia salmositica* (blood form). Agglutination by lectins after treatment with neuraminidase (NT)

Lectin	Addition	Lectin concentration (μg ml^{-1}) 0.0	1.0	2.0	4.0	8.0	16	32	64	128	256
Con A	None	–	+	2+	3+	4+	4+	4+	4+	4+	4+
	NT	–	+	+	2+	3+	3+	4+	4+	4+	4+
ECA	None	–	–	–	–	–	–	–	–	–	–
	NT	–	–	–	–	–	–	–	+	+	+
HPA	None	–	–	–	–	–	–	–	–	–	–
	NT	–	–	–	–	–	–	–	–	+	+
LCA	None	–	–	–	–	–	–	–	–	–	–
	NT	–	–	–	–	–	–	–	–	+	+
PCA	None	–	–	–	–	–	–	–	–	–	–
	NT	–	–	–	–	–	–	–	–	–	–
PWM	None	–	–	–	–	–	–	–	–	–	+
	NT	–	–	–	–	–	–	–	–	–	–
SNA	None	–	–	–	–	–	–	–	–	–	–
	NT	–	–	–	–	–	–	–	–	–	+
TPA	None	–	–	–	–	–	–	–	–	–	–
	NT	–	–	–	–	–	–	–	–	–	–
MAA	None	–	–	–	–	–	–	–	–	–	–
	NT	–	–	–	–	–	–	–	–	–	–
WGA	None	–	–	–	–	–	–	–	–	–	–
	NT	–	–	–	–	–	–	–	–	+	+
PNA	None	–	–	–	–	–	–	–	–	–	–
	NT	–	–	–	–	–	–	–	–	–	–
PSA	None	–	–	–	–	+	+	+	2+	2+	3+
	NT	–	–	+	+	+	+	2+	2+	2+	3+

Table 4. *Cryptobia salmositica* (blood form). Agglutination by lectins after treatment with trypsin (TT)

Lectin	Addition	Lectin concentration (μg ml^{-1}) 0.0	1.0	2.0	4.0	8.0	16	32	64	128	256
Con A	None	–	+	2+	3+	4+	4+	4+	4+	4+	4+
	TT	–	+	+	+	+	2+	2+	2+	2+	3+
ECA	None	–	–	–	–	–	–	–	–	–	+
	TT	–	–	–	–	–	–	–	–	–	–
HPA	None	–	–	–	–	–	–	–	–	–	–
	TT	–	–	–	–	–	–	–	–	–	–
LCA	None	–	–	–	–	–	–	–	–	–	–
	TT	–	–	–	–	–	–	–	–	+	+
PCA	None	–	–	–	–	–	–	–	–	–	–
	TT	–	–	–	–	–	–	–	–	+	+
PWM	None	–	–	–	–	–	–	–	–	–	+
	TT	–	–	–	–	–	–	–	–	–	–
SNA	None	–	–	–	–	–	–	–	–	–	–
	TT	–	–	–	–	–	–	–	–	–	–
TPA	None	–	–	–	–	–	–	–	–	–	–
	TT	–	–	–	–	–	–	–	–	–	–
MAA	None	–	–	–	–	–	–	–	–	–	–
	TT	–	–	–	–	–	–	–	–	–	–
WGA	None	–	–	–	–	–	–	–	–	–	–
	TT	–	–	–	–	–	–	–	–	–	–
PNA	None	–	–	–	–	–	–	–	–	–	–
	TT	–	–	–	–	–	–	–	–	–	–
PSA	None	–	–	–	–	+	+	+	2+	2+	3+
	TT	–	–	–	–	+	+	+	+	+	2+

Treatment of cell surface of *Cryptobia bullocki* with neuraminidase greatly enhanced agglutination of the parasite with lectins ECA, HPA, LCA, SNA, TPA, MAA, WGA, PNA and PSA. Only the agglutination with PCA was eliminated (Table 5). After trypsin treatment, agglutination with PCA, SNA, WGA, PNA and PSA was enhanced, while that with Con A, ECA, HPA was diminished. No agglutination was mediated by MAA (Table 6).

Terminal sugar and linkage differences in carbohydrate surface glycoprotein between *Cryptobia* spp.

The terminal sugar of surface carbohydrate on the 3 species of *Cryptobia* was examined using 12 lectins and the results showed there were differences in terminal sugars. PCA, Con A and TPA had unique staining patterns with the parasites (Fig. 1A, B, C); however, HPA and ECA (Fig. 1D), LCA and PSA (Fig. 1E), WGA and PWM (Fig. 1F) showed similar staining patterns with another lectin. Nevertheless, they all showed differences between species of *Cryptobia*.

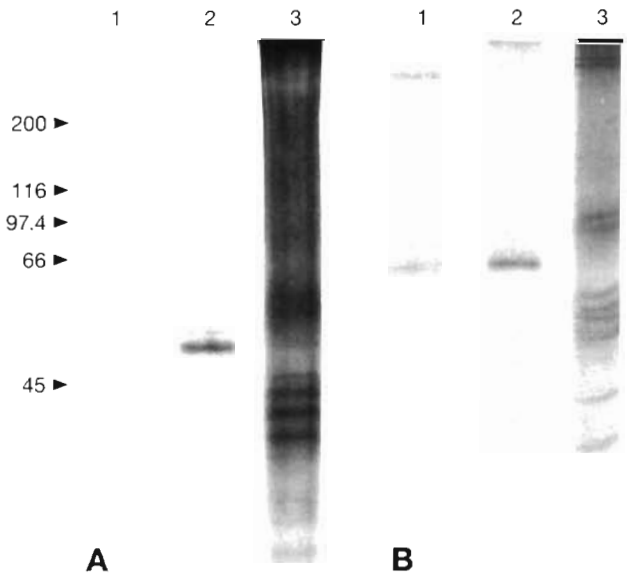


Fig. 1 *Cryptobia salmositica*, *C. bullocki* and *C. catostomi*. Detection of glycoproteins using biotin-labelled lectins. (A) PCA, (B) Con A, (C) TPA, (D) HPA/ECA, (E) LCA/PSA and (F) WGA/PWM. Lane 1, *C. salmositica*; lane 2, *C. bullocki*; lane 3, *C. catostomi*. Numbers on the left indicate molecular weights of standards

Table 5. *Cryptobia bullocki* (culture form). Agglutination by lectins after treatment with Neuraminidase (NT)

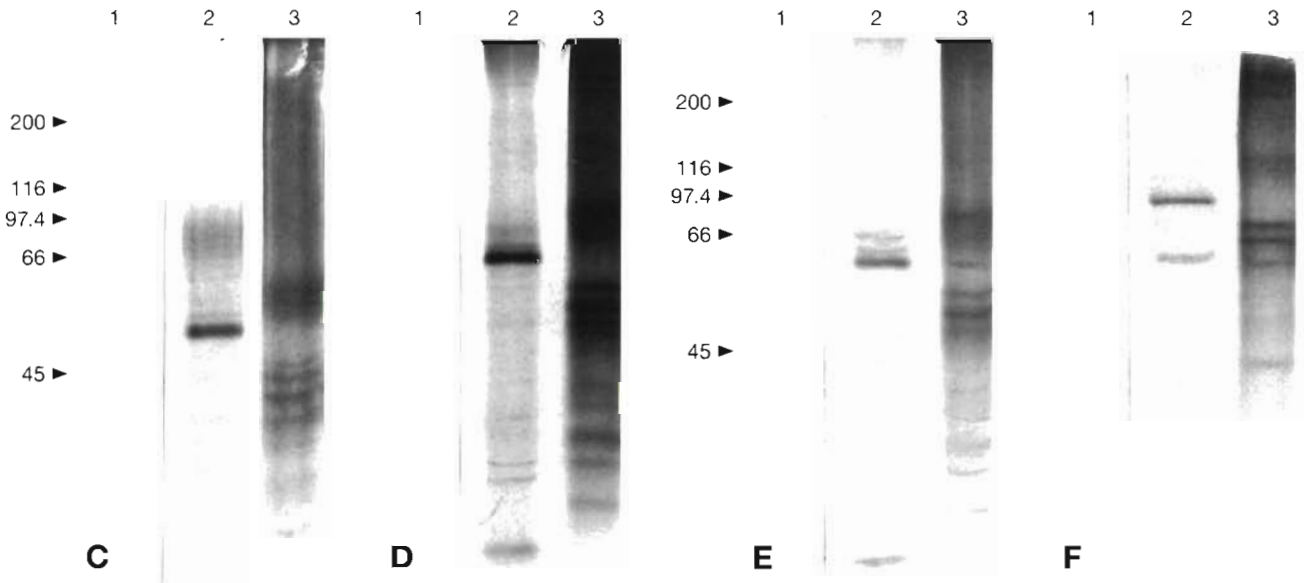
Lectin	Addition	Lectin concentration (μg ml^{-1}) 0.0	1.0	2.0	4.0	8.0	16	32	64	128	256
Con A	None	–	+	2+	3+	4+	4+	4+	4+	4+	4+
	NT	–	+	+	2+	2+	2+	3+	3+	4+	4+
ECA	None	–	–	–	–	–	–	–	+	+	+
	NT	–	–	–	–	+	+	+	+	2+	2+
HPA	None	–	–	–	–	–	–	–	+	+	+
	NT	–	–	–	+	+	+	+	2+	2+	2+
LCA	None	–	–	–	–	–	–	–	+	+	2+
	NT	–	–	–	–	–	+	+	+	2+	2+
PCA	None	–	–	–	–	–	–	–	–	–	+
	NT	–	–	–	–	–	–	–	–	–	–
PWM	None	–	–	–	–	–	–	–	–	–	–
	NT	–	–	–	–	–	–	–	–	–	–
SNA	None	–	–	–	–	–	–	–	–	–	–
	NT	–	–	–	–	–	–	–	+	+	+
TPA	None	–	–	–	–	–	–	–	–	–	–
	NT	–	–	–	–	+	+	+	+	2+	2+
MAA	None	–	–	–	–	–	–	–	–	–	–
	NT	–	–	–	+	+	+	+	2+	2+	2+
WGA	None	–	–	–	–	–	–	–	–	+	+
	NT	–	–	–	+	+	+	+	2+	2+	2+
PNA	None	–	–	–	–	–	–	–	–	–	–
	NT	–	–	+	+	+	+	+	2+	2+	2+
PSA	None	–	–	–	–	–	–	–	+	+	+
	NT	–	–	–	+	+	+	+	2+	2+	2+

Table 6. *Cryptobia bullocki* (culture form). Agglutination by lectins after treatment with trypsin (TT)

Lectin	Addition	Lectin concentration (μg ml^{-1}) 0.0	1.0	2.0	4.0	8.0	16	32	64	128	256
Con A	None	–	+	2+	3+	4+	4+	4+	4+	4+	4+
	TT	–	+	+	2+	2+	2+	3+	3+	4+	4+
ECA	None	–	–	–	–	–	–	–	+	+	+
	TT	–	–	–	–	–	–	–	–	–	+
HPA	None	–	–	–	–	–	–	–	+	+	+
	TT	–	–	–	–	–	–	–	–	–	–
LCA	None	–	–	–	–	–	–	–	+	+	2+
	TT	–	–	–	–	–	–	–	–	–	–
PCA	None	–	–	–	–	–	–	–	–	–	+
	TT	–	–	–	–	–	–	–	–	+	+
PWM	None	–	–	–	–	–	–	–	–	–	–
	TT	–	–	–	–	–	–	–	+	+	+
SNA	None	–	–	–	–	–	–	–	–	–	–
	TT	–	–	–	–	–	–	–	–	–	+
TPA	None	–	–	–	–	–	–	–	–	–	–
	TT	–	–	–	–	–	–	–	–	–	+
MAA	None	–	–	–	–	–	–	–	–	–	–
	TT	–	–	–	–	–	–	–	–	–	–
WGA	None	–	–	–	–	–	–	–	–	+	+
	TT	–	–	–	+	+	+	+	2+	2+	2+
PNA	None	–	–	–	–	–	–	–	–	–	–
	TT	–	–	+	+	+	+	2+	2+	2+	2+
PSA	None	–	–	–	–	+	+	+	+	+	+
	TT	–	–	–	+	+	+	+	2+	2+	2+

Most of the lectins (GNA, PNA, Con A, PCA, TPA, HPA, ECA, LCA, PSA, WGA and PWM) showed a species-specific staining pattern (Fig. 1A to F; Fig. 2A, D) except SNA and MAA. SNA [for sialic acid linked α(2-6) or α(2-3) to galactose] showed the same staining pattern among the 3 species (Fig. 2B) and MAA showed the same staining pattern between *Cryptobia salmositica* and *C. bullocki* but a different pattern in *C. catostomi* (Fig. 2C).

Fewer glycoconjugates were detected by the lectins in pathogenic *Cryptobia salmositica* than in the culture form of *C. bullocki* and non-pathogenic *C. catostomi*.



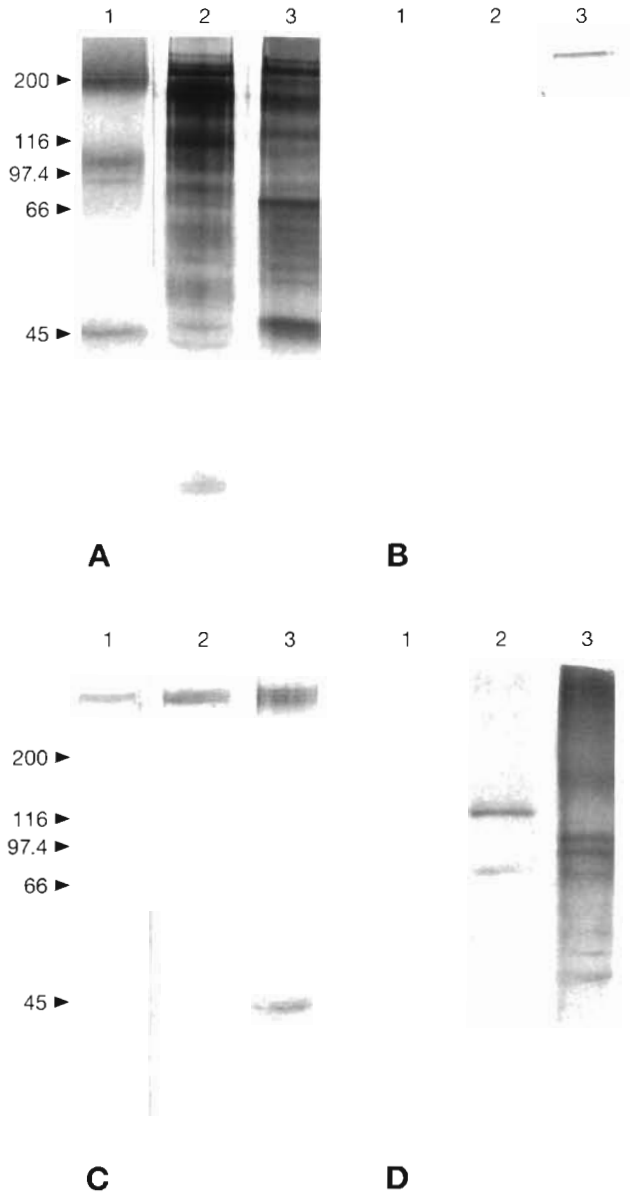


Fig. 2. *Cryptobia salmositica*, *C. bullocki* and *C. catostomi*. Detection of glycoproteins with digoxigenin-labelled lectins. (A) GNA, (B) SNA, (C) MAA and (D) PNA. Lane 1, *C. salmositica*; lane 2, *C. bullocki*; lane 3, *C. catostomi*. Numbers on the left indicate molecular weights of standards

Only 4 of 13 lectins (Con A, GNA, SNA and MAA; Fig. 1B, lane 1 & Fig. 2A, B, C, lane 1) detected single or 4 components of glycoconjugate in *C. salmositica* and no reactions were observed with the other lectins (PCA, TPA, HPA, ECA, LCA, PSA, WGA, PWM and PNA, in Fig. 1A, C, D, E, F, Fig. 2D). There were few components of glycoconjugates detected by PWM, WGA, LCA, PSA, Con A, PCA, PNA, SNA and MAA (Figs. 2B, C, D & 1A, B, E, F), but many components of glycoconjugates in a wide range (20 to 220 kDa) with GNA, TPA, HPA and ECA (Figs. 2A & 1C, D) in the culture form of *C. bullocki*. In contrast to the result in *C. bullocki*, most of the lectins (PCA, Con A, TPA, HPA, ECA, LCA, PSA, WGA, PWM, GNA, PNA) detected multiple components of glycoconjugates with relative molecular masses ranging from 25 to 220 kDa (Fig. 1A, B, C, D, E, F, Fig. 2A & D, lane 3) and only 2 lectins (SNA, MAA) detected single component or 2 triplets at 45 and 220 kDa (Fig. 2B & C, lane 3) in *C. catostomi*.

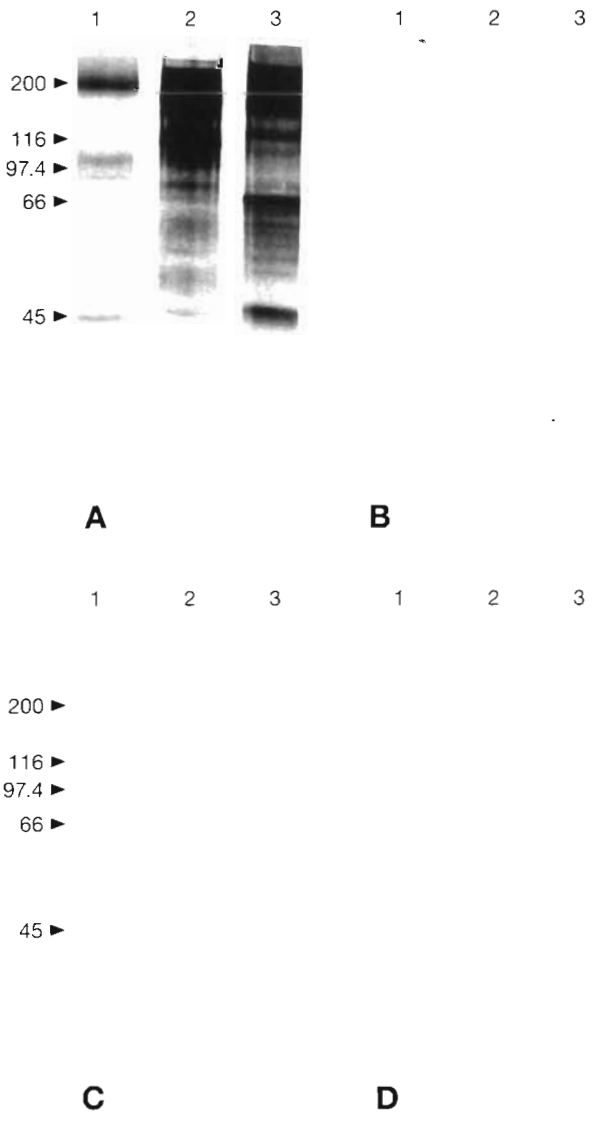


Fig. 3. *Cryptobia salmositica*, *C. bullocki* and *C. catostomi*. Identification of carbohydrate linkage on glycoprotein with high mannose components using glycosidases. Glycoproteins digested with (A) N-glycosidase F, (B) endoglycosidase F, (C) endoglycosidase H and (D) without digestion. Lane 1, *C. salmositica*, lane 2, *C. bullocki*; lane 3, *C. catostomi*. Numbers on the left indicate molecular weights of standards

Deglycosylation with glycosidase showed that there were N-linked carbohydrates containing mannose on the surface coat of *Cryptobia salmositica*, *C. bullocki* and *C. catostomi* because there were no reactions with lectin GNA after digestion with N-glycosidase F, endoglycosidase F and endoglycosidase H (Fig. 3). O-glycans were not detected on the surface coat of the 3 species.

DISCUSSION

Lectin-mediated agglutinations showed differences between pathogenic and nonpathogenic cryptobiids. Pathogenic *Cryptobia salmositica* was agglutinated by 3 lectins; culture form *C. bullocki* was agglutinated by 5 further lectins. The nonpathogenic *C. catostomi* was agglutinated by most of the lectins (10 lectins). Con A and PSA, which are specific for α-D-mannose agglutinated all 3 species and required very similar concentrations of lectins. This indicates that mannose moieties on the surface membranes of the 3 species are similar and confirms that the species are taxonomically related. Lectin ECA, HPA, LCA, PCA and SNA agglutinated both *C. bullocki* and *C. catostomi* but lower concentration of lectins are required by *C. catostomi*. This indicates that β-gal-(1-4)gal, galNAc, α-man and α-neuNAc(2-6)gal/galNAc moieties are on both *C. bullocki* and *C. catostomi*, but there are more moieties on nonpathogenic *C. catostomi* than *C. bullocki*. Since these carbohydrate moieties, except α-D-mannose, were not detected on pathogenic *C. salmositica*, they could not be related to pathogenicity. *C. bullocki* is a pathogenic haemoflagellate in summer flounders *Paralichthys dentatus* Linnaeus on the Atlantic coast of North America and Gulf of Mexico (Burreson 1982). However, the culture strain we have is not likely to be pathogenic as it has been maintained in culture since 1987 (Woo 1987). We assume the pathogenicity of our strain of *C. bullocki* has been reduced or lost during the *in vitro* culture as with the culture strain of *C. salmositica* (see Woo & Li 1990, Zuo & Woo 1997).

The binding to lectins was affected by enzyme treatment. In all 3 species, the binding of some lectins was increased with enzyme treatment while there were reductions in some of them after enzyme treatment. The increase in agglutination after enzyme treatment might be due to exposure of some residues such as galNAc-like residues on the cell surface, or to exclusively greater exposition of the adjacent carbohydrate, or to a combination of these effects and the decrease in the superficial negative charge, thus reducing the superficial repulsion between cells (Nicolson 1973). However, the decrease in binding might be because protein or carbohydrate moieties were removed or degraded by the enzyme. Con A-mediated agglutination of *Cryptobia* was decreased after treatment with trypsin, which indicates that Con A binding sites are attached to proteins rather than to lipids. Since WGA can bind to either sialic acid or glcNAc-containing residues, WGA-mediated agglutination of *Cryptobia* was enhanced after treatment with neuraminidase indicating that more glcNAc-like residues were exposed by the removal of sialic acids. Similarly, HPA-, PWM- and SNA-mediated agglutinations were enhanced by the same mechanism after treatment with neuraminidase.

Although lectin-mediated agglutination indicates carbohydrate residues on cells, the absence of agglutination does not necessarily mean the absence of particular carbohydrates. Lectin blotting has been used both on purified membrane proteins (Jaffe & McMahon-Pratt 1988) and crude membrane extracts of *Leishmania* promastigotes (Grogl et al. 1987, Rossell et al. 1990) to compare the membrane glycoconjugate components. In present study, SNA and MAA did not agglutinate *Cryptobia salmositica* but detected a glycoprotein band of high molecular weight in lectin blot; PWM, MAA and PNA also did not agglutinate *C. bullocki* but detected glycoproteins on *C. bullocki*. The culture form of *C. bullocki* and the nonpathogenic species *C. catostomi* reacted with all lectins and *C. catostomi* had the strongest reactions among the 3 species. Although the lanes of *C. catostomi* in the figures look overloaded, they had the same amount of protein as the other samples. The present study confirms that lectin blot is more sensitive than lectin-mediated agglutination in detecting surface carbohydrate moieties. However, it is also possible that some cytoplasmic carbohydrates contaminated the Triton extract and reacted with the lectins in the lectin blots.

Lectin blot of surface glycoconjugates showed increased expression of surface carbohydrates in the culture form of *Cryptobia bullocki* and the nonpathogenic *C. catostomi*. β-gal(1-4)gal, galNAc, α-man, α-NeuNAc(2-6)gal/galNAc were detected on the surface of *C. bullocki* and *C. catostomi* but not in *C. salmositica* using lectin-mediated agglutination. The surface carbohydrate expression of *Leishmania major* was affected by the nutrient contents in the culture medium and by the diet of vectors and the parasite adapts either by selection or changes metabolically in the culture or vector (Wallbanks et al. 1986, Schlein 1987). Studies on the virulent and avirulent strains of *C. salmositica* suggested that the surface carbohydrates had changed during continuous *in vitro* culture. The attenuated avirulent strain developed more surface carbohydrates and at the same time lost metalloprotease which is present in the virulent strain and this may be related to the pathogenicity of *C. salmositica* (see Zuo & Woo 1997) during *in vitro* culture. We also

believe that the surface carbohydrate expression on *C. bullocki* was changed by continuous *in vitro* culture and further comparison of the blood form and culture form of *C. bullocki* may reveal the changes.

Surface-exposed sialic acid residues were demonstrated on *Cryptobia salmositica*, *C. bullocki* and *C. catostomi* using lectin-mediated agglutination and lectin blot. The nonpathogenic *C. catostomi* and culture strain of *C. bullocki* expressed more sialic acid than pathogenic *C. salmositica*. Vommaro et al. (1997) demonstrated surface-exposed sialic acid residues on *C. salmositica* and *C. bullocki* and they contributed to the surface charge. The sialic acid residues on *Trypanosoma cruzi* and bodonids (Vommaro et al. 1993) also contributed significantly to their negative surface charge. Surface-exposed sialic acids were assumed to play important roles as biological masks (Schauer et al. 1983) which protect *T. cruzi* blood forms from complement-mediated lysis (Kipnis et al. 1981) and phagocytosis by macrophages (Araujo-Jorge & De Souza 1984). Lectins ECA, HPA, SNA and PNA agglutinated *C. catostomi* at a lower concentration and the culture form of *C. bullocki*. No agglutination was seen in pathogenic *C. salmositica*. This suggests that nonpathogenic *C. catostomi* expressed more exposed galactose-like or N-acetylgalactosamine-like residues than the culture form of *C. bullocki*. Pathogenic *C. salmositica* did not have enough exposed galactose-like or N-acetylgalactosamine-like residues on the surface to be bound by lectin. This is similar to the non-infective promastigotes stage of *Leishmania major* while infective promastigotes lose their PNA binding sites (Sacks et al. 1985). However, the function of sialic acids and galactose-like or N-acetylgalatosamine-like residues on non-pathogenic *Cryptobia* is not fully understood.

GNA, Con A and LCA lectins with the same major specificities did not bind to the same glycoproteins on *Cryptobia salmositica*. This suggests differences in accessibility of binding sites. GNA binds strongly to the largest number of glycoproteins and this is probably related to its specificity for the more common sugars and its ability to bind to internal and terminal sugars. The observation supports previous work (Macgregor et al. 1985) on the lectin-binding glycoprotein on the surface of *Schistosoma mansoni*.

Con A killed *Cryptobia* spp. at the concentration where agglutination occurred. All other lectins used in the present study were not toxic to these parasites. The cytotoxic effect of lectins on leishmanial parasites is known. Leishmanial cells were agglutinated by RCA-I and the toxic effect was not reversible (Jacobson et al. 1982). Zajicek & Peckova (1990) also observed decreased cell mobility of freshwater fish trypanosomes in LCA and WGA lectins, but these had no visible effects on our *Cryptobia*.

Our study clearly indicates that pathogenic and nonpathogenic *Cryptobia* spp. differ in their surface carbohydrate components. Since oligosaccharides on and within the surface membrane of the parasites are the keys to their survival in their host or in culture, this comparative study on surface carbohydrates may lead us to a better understanding of the pathogenic mechanism and perhaps a more rational approach to the treatment of cryptobiosis.

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